Regulation of Aflatoxin Biosynthesis: Assessment of the Role of Cellular Energy Status as a Regulator of the Induction of Aflatoxin Production

ROBERT L. BUCHANAN,* SUSAN B. JONES, WALTER V. GERASIMOWICZ, LAURA L. ZAIKA, HEIDI G. STAHL, AND LORI A. OCKER

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

Received 22 December 1986/Accepted 9 March 1987

Possible relationships among cellular energy status and the induction and initiation of aflatoxin synthesis were studied by using replacement culture techniques in conjunction with aflatoxin-supporting and-nonsupporting media. Transcription and translation processes associated with the induction of aflatoxin synthesis occurred 3 to 6 and 6 to 10 h, respectively, after mycelia were transferred to glucose-containing media. From adenylate energy charge determinations and in situ ^{31}P nuclear magnetic resonance analyses, a relationship between overall energy status and the induction or initiation of aflatoxin synthesis could not be identified; however, electron microscopic evaluations indicated that aflatoxin synthesis occurred in association with a glucose-mediated inactivation of mitochondria. The results suggest that aflatoxin synthesis is not regulated by the overall energy status of the fungal cell but may be controlled by the energy status of specific subcellular compartments.

A number of investigators have observed that the production of aflatoxins is highly dependent on the identity (2, 8, 9, 17, 20) and concentration (3, 5, 10, 11, 15, 17, 24) of available carbohydrates or related carbon sources. Abdollahi and Buchanan (1, 2) developed a nutritional shift protocol to study this effect based on the observation that *Aspergillus parasiticus* is not capable of synthesizing aflatoxins when cultured on a peptone-mineral salts medium (PMS) but is induced to produce the toxins if transferred to a carbohydrate-containing medium. Results of that study and subsequent investigations in our laboratory have suggested that aflatoxin biosynthesis may be regulated by a carbon catabolite induction process, in which the buildup of a key intermediate or alterations in the energy status of the cell leads to the induction of aflatoxin synthesis (1, 2, 6–8). It has also been determined that a nutritional shift from a peptone-based medium to one containing a carbohydrate source (thereby inducing aflatoxin synthesis) alters the activities of various glycolytic and tricarboxylic acid cycle enzymes, again suggesting that alterations in the energy status of the mold may influence its ability to synthesize aflatoxins (4, 6, 7).

The objective of this study was to evaluate possible relationships among the bioregulation of aflatoxin production and the energy status of *A. parasiticus* by using the aforementioned peptone-to-glucose nutritional shift protocol. Energy status was monitored by using both energy charge determinations and in situ high-resolution nuclear magnetic resonance (NMR) analysis of phosphorus compounds. Additionally, studies of the ultrastructural changes that occurred as a result of the nutritional shift were performed.

* Corresponding author.

MATERIALS AND METHODS

Microorganism. *A. parasiticus* NRRL 2999 was used throughout the study. Stock cultures were maintained on potato glucose agar (Difco Laboratories, Detroit, Mich.) slants incubated at 28°C until they were well sporulated and then were stored at 4°C. Spore suspensions were prepared by adding 5 ml of sterile water with Tween 80 (20 μl/100 ml) to a stock slant and agitating gently. The harvested spores were subsequently enumerated by using a hemacytometer and diluted to 10^6 conidia per ml.

Media. Yeast extract-sucrose medium (YES) was prepared as described by Davis et al. (11). Yeast extract-peptone medium (YEP) was prepared by replacing the sucrose in YES with 60 g of peptone. PMS and glucose-mineral salts medium (GMS) were prepared by the method described by Buchanan and Lewis (6). Peptone-glucose-mineral salts medium (PGMS) was prepared by adding 60 g of glucose to 1,000 ml of PMS. The initial pHs of the media were adjusted to the desired values by using 10 N HCl. Unless otherwise specified, all media were sterilized by autoclaving at 15 lb/in^2 for 15 min.

Modified PMS, GMS, and PGMS were employed as the final replacement media for the ^{31}P NMR studies. The modification consisted of omitting the KH_2PO_4 and altering the stock solution of trace metals such that it contained the following per 1,000 ml of distilled and deionized water; 1.76 g of $ZnSO_4 \cdot 7H_2O$, 100 mg of $Fe_2(SO_4)_2 \cdot 6H_2O$, 70 mg of $Na_2MoO_4 \cdot 2H_2O$, 70 mg of $Na_2B_4O_7 \cdot 10H_2O$, and 30 mg of $CoSO_4 \cdot 4H_2O$.

Transcription and translation inhibitor studies. The time requirements for the transcription and translation processes that were associated with the induction of aflatoxin synthesis after transfer of the mold from an aflatoxin-nonsupporting to an aflatoxin-supporting medium were studied by the technique described by Abdollahi and Buchanan (1) which was

modified to employ the nutritional shift replacement culture protocol described by Buchanan and Lewis (6). The mold was initially cultured in 1,000-ml flasks containing 300 ml of YES. The flasks were inoculated to a level of 7×10^3 conidia per ml and incubated for 72 h at 28°C on a rotary shaker (150 rpm). The mycelial pellets were harvested on cheesecloth, rinsed with sterile 0.85% KCl, and disrupted in a blender along with 450 ml of sterile 0.85% KCl. The mycelia were then transferred to 1,000-ml flasks with 300 ml of PMS (pH 4.5) and incubated for 24 h at 28°C and 150 rpm. PMS does not induce or support the synthesis of aflatoxins, and during this period of incubation the mold reverts to an aflatoxin-nonproducing state (4, 6, 7).

The mycelia were collected on cheesecloth, rinsed with 0.85% KCl, and transferred in 1.0-g (wet weight) portions to 50-ml flasks containing 10 ml of GMS (pH 5.5), which does induce and support the formation of aflatoxins. These flasks were incubated at 28°C and 100 rpm. At designated times up to 12 h posttransfer, six replicate cultures were removed, with three being immediately analyzed for pH of the media, aflatoxin production, and mycelial dry weight. The other three flasks were supplemented with either cycloheximide or actinomycin D to a level of 200 or 150 μg/ml, respectively. The cultures were incubated at 28°C and 100 rpm until 48 h posttransfer, at which time they were analyzed for pH of the media, aflatoxin production, and mycelial dry weight.

^{31}P NMR study. The status of phosphate pools in *A. parasiticus* was studied by ^{31}P NMR analysis by using a modification of the replacement culture protocol described above. After sequential culturing in YES and PMS, thoroughly rinsed mycelia were transferred in 5-g portions to 125-ml flasks containing 25 ml of modified GMS, PMS, or PGMS. The pHs of these media were 4.5. The flasks were incubated at 28°C on a rotary shaker (150 rpm). After 0, 1, 2, 4, 6, 24, and 30 h of incubation, a set of cultures was harvested and the media were frozen (−20°C) for later ^{31}P NMR analysis. The mycelia were rinsed thoroughly with double distilled and deionized water. For each culture, a 1.0-g portion of mycelium was transferred to a NMR sample tube (10 mm) containing 3 ml of double distilled and deionized water. A perfusion-type vortex plug with a reference capillary was inserted, and the tube was sealed with a plastic cap. The mycelium was then dispersed evenly within the designated volume of the tube by agitating on a vortex mixer. After mixing the tube was placed in a sample holder and immediately inserted into the NMR spectrometer. The elapsed time between harvesting of the culture and insertion was less than 2 min. Samples of the collected replacement media plus samples of uninoculated media were also analyzed by transferring 3.0-ml portions into NMR sampling tubes. A duplicate set of cultures was analyzed for aflatoxin production, pH of the media, and mycelial dry weight.

^{31}P NMR spectra were acquired at 161.7 MHz with an NMR spectrometer system (JEOL JNM-GX 400 FT) that included a 9.4-T narrow bore (54 mm; Oxford) magnet and a computer system (DEC LSI 11/23). Measurements were carried out at 28 ± 1°C. A 16,000-Hz frequency range was examined with 2,000 data points that were zero filled to 16,000 ^{31}P chemical shifts were externally referenced to 0.120 M hexamethylphosphoramide (HMPA) in a sealed capillary placed in the center of the NMR tube. The HMPA was assigned a value of 13.78 ppm for its resonance position so that the ^{31}P chemical shifts in this study would be consistent with those assigned previously (19, 22). The spectra were rapidly acquired with 5,000 transients per accumulation and a repetition time of 0.162 s (total time for accumulation of each spectrum was approximately 13.5 min). A 30° pulse (12 μs) without broadband decoupling was used. ^{31}P chemical shifts of P_i relative to the chemical shift of HMPA at 13.78 ppm were used to evaluate intracellular pH changes. HMPA itself shows no chemical shift dependence as a function of pH and is stable for long periods over wide temperature ranges.

Electron microscopy. After transfer to aflatoxin-supporting and -nonsupporting media, the ultrastructure of *A. parasiticus* was studied by using the replacement culture technique. After sequential culturing in YES and PMS, 10-g portions of the mycelia were transferred to 125-ml flasks containing 25 ml of PMS, GMS, or PGMS (pH 4.5) and incubated without agitation at 28°C. After 0, 6, 24, and 48 h of incubation, a culture of each medium was harvested and samples of the mycelia were fixed for 2 to 3 h at room temperature with 2% glutaraldehyde in 0.08 M sodium cacodylate buffer (pH 7.0). The samples were then rinsed three times with the same buffer in conjunction with harvesting by light centrifugation with a bench top clinical centrifuge. The loose cell pellet, measuring approximately 0.3 ml, was mixed with 2 to 3 drops of molten 2% agar (in the same buffer) at 42°C. The mixture was pipetted onto a clean glass slide, allowed to harden for 3 to 4 min, and cut into 2-mm^3 blocks. The blocks were subsequently postfixed with 1% osmium tetroxide in the same buffer for 3 h, dehydrated with a graded water-acetone series, and embedded in Spurr low-viscosity resin. Ultrathin (60 to 70-nm) sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed in a transmission electron microscope (EM 10; Zeiss) operated at 60 kV. A duplicate set of cultures was analyzed for aflatoxin production, pH of the media, and mycelial dry weights.

To relate the changes that were observed in the replacement culture mycelia with those that occurred during the normal growth cycle of the mold, additional samples for electron microscopic evaluation were generated by using conidia-initiated cultures. Flasks (125 ml) containing 25 ml of YES or YEP were inoculated with 2.0 ml of spore suspension to achieve an inoculum of 4×10^5 conidia per ml. The flasks were incubated at 28°C without agitation. After 24, 48, and 72 h, the cultures were harvested and the mycelial mat was sampled in at least two locations. The samples were immediately fixed as described above.

Determination of adenine nucleotides. The mold was sequentially cultured in YES and PMS as described above and was transferred in 2.0-g (wet weight) portions to 50-ml flasks containing 15 ml of PMS, GMS, or PGMS and incubated at 28°C on a rotary shaker (150 rpm). After 0, 3, 5.5, 24, 28, 48, and 52 h of incubation, the mycelium was harvested, rinsed thoroughly and rapidly with 0.85% KCl, blotted to remove excess moisture, and weighed; and adenine nucleotides were extracted by using a modification of the technique described by Khym (16).

All steps of the extraction were carried out at 5°C in a cold room. Mycelium was transferred to 30-ml plastic screw-cap centrifuge tubes containing 5 ml of cold 0.5 M trichloroacetic acid. The centrifuge tubes were shaken vigorously for 30 s and then placed on a wrist-action shaker for 30 min. After centrifugation (10 min at 13,000 × *g*), the supernatant was poured through a cheesecloth plug to remove insoluble material. Excess acid was removed by extracting 1 ml of the filtrate with 1 ml of Freon (1,1,2-trichlorotrifluoroethane; E. J. du Pont de Nemours & Co., Inc., Wilmington, Del.) containing 0.5 M tri-*n*-octylamine. The mixture was shaken vigorously for 30 s and allowed to stand until separation of

TABLE 1. Aflatoxin production by *A. parasiticus* after transfer to different replacement culture media[a]

Replacement culture medium	Posttransfer incubation time (h)	Mycelium dry wt (g)	pH[b]	Amt of aflatoxin in culture (μg)
GMS	6	1.19	5.3	ND[c]
	24	1.39	3.6	23.5
	48	1.48	3.0	108.3
PGMS	6	1.44	5.0	ND
	24	1.56	4.6	23.5
	48	1.79	5.4	100.1
PMS	6	1.21	5.2	ND
	24	1.29	6.5	ND
	48	1.17	7.5	ND

[a] Mold was sequentially cultured in YES and PMS and then transferred in 10-g portions (wet weight) to 125-ml flasks containing 25 ml of medium.
[b] Initial pH was 5.5.
[c] ND, None detected.

the two phases. The upper (aqueous) layer was clarified by centrifugation, and the supernatant was frozen at −20°C.

Adenine nucleotides were separated and quantitated by high-pressure liquid chromatography with a gradient liquid chromatograph (model 332; Beckman Instruments, Inc. Fullerton, Calif.) that incorporated two pumps (model 110A; Beckman), a controller (model 421; Beckman), a sample injector (model 210; Beckman). The UV detector (model 1203; Laboratory Data Control) was equipped with a 10-μl and 10-mm light path flow cell and a 254-nm filter and was interfaced with an electronic integrator (model 3390A; Hewlett-Packard Co., Palo Alto, Calif.).

Mycelial extracts (10 μl) were separated on an Ultrasphere IP column (4.6 by 250 mm; Altex) protected by an Ultrasphere IP guard column (4.6 by 40 mm; Altex). ADP and ATP were separated in the ion pair mode by using isocratic elution with 0.01 M tetrabutylammonium phosphate in 0.08 M KH_2PO_4 (pH 6.5) with 12% acetonitrile. AMP was determined chromatographically in the reversed-phase mode by using isocratic elution with 0.1 M KH_2PO_4 (pH 5.0)–1.5% acetonitrile. The flow rate in both cases was 1 ml/min. Concentrations of AMP, ADP, and ATP in mycelial extracts were determined by comparing their peak heights with those of external standards.

Aflatoxin analyses and determination of mycelial dry weights. Aflatoxin production was determined by extracting the cultures (mycelium and medium) with chloroform, separating the toxins by thin-layer chromatography, and quantitating the toxins by fluorodensitometry as described previously (7). After extraction the mycelia were collected on preweighed filter paper, rinsed thoroughly with distilled water, and then dried for 18 h at 85°C. Mycelial dry weights were then determined gravimetrically.

RESULTS AND DISCUSSION

Before the technique described by Buchanan and Lewis (6) was employed to study the role of cellular energy status on the induction of aflatoxin production, a series of studies was performed to confirm the comparability of the protocol with that employed by Abdollahi and Buchanan (1), particularly with regard to the use of disrupted mycelial pellets for determining the timing of the transcription and translation processes associated with the induction of aflatoxin synthesis by glucose. In Table 1 the typical response observed when *A. parasiticus* was sequentially cultured in YES and PMS and then transferred to PMS, GMS, and PGMS is shown. Aflatoxin production was not observed in PMS, while equivalent levels of toxin production occurred in GMS and PGMS. This agrees with the results of Abdollahi and Buchanan (1) and indicates that aflatoxin synthesis is regulated by a carbohydrate-mediated induction and is not due to derepression by the removal of peptone.

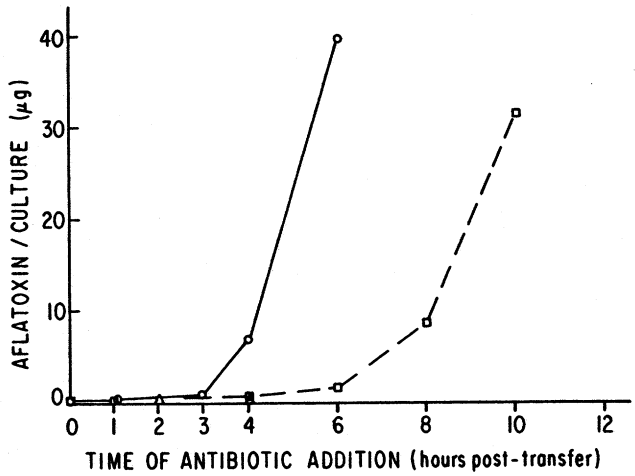

FIG. 1. Time requirements for the transcription and translation processes associated with the induction of aflatoxin synthesis by glucose after the transfer of mycelia from PMS to GMS. Actinomycin D (○) and cycloheximide (□) were added after various periods of posttransfer incubation, with cultures being analyzed for aflatoxins after 48 h of total incubation. Values are means of at least three replicate cultures.

Based on the use of transcription and translation inhibitors, Abdollahi and Buchanan (1) have concluded that when intact mycelial pellets are transferred from PMS to GMS, a period of de novo gene expression is required for the initiation of aflatoxin synthesis. They estimated that approximately 6 h are required for this process. This result was reexamined by using the replacement culture protocol described by Buchanan and Lewis (6) to define more carefully the time requirements for the glucose-mediated induction of aflatoxin synthesis by utilizing the mold is a filamentous form. After the mold was transferred from PMS to GMS, the cultures were supplemented with actinomycin D or cycloheximide after various time intervals to block transcription and translation, respectively. Incubation was continued until 48 h posttransfer to allow the cultures to produce aflatoxins if they had achieved that capability. Additional cultures were examined at the time of antibiotic addition to determine when toxin synthesis was actually initiated.

No de novo aflatoxin production was observed over the 48-h post transfer incubation period if actinomycin D was added before 3 h of incubation (Fig. 1). However, addition of the antibiotic at >3 h allowed increasing amounts of toxin formation, and by 6 h the addition of actinomycin D no longer affected toxin formation during the posttransfer incubation period. A similar pattern was observed with cycloheximide, except that attainment of the ability to produce toxin was displaced further in time. Aflatoxin production was prevented by cycloheximide when cycloheximide was added before 6 h, while increasing amounts of toxin accumulation were observed when cycloheximide additions were made after 6 to 10 h of posttransfer incubation. Analyzing

TABLE 2. Adenine nucleotide and energy charge levels of mycelia of *A. parasiticus* replacement cultures[a]

Replacement medium	Posttransfer incubation time (h)	Adenine nucleotides/mycelium (nmol/g [wet wt] of mycelium)[b]			
		AMP	ADP	ATP	Energy charge[c]
	0	111 (9)	102 (3)	276 (6)	0.67
PMS	3	51 (1)	61 (2)	184 (40)	0.73
	6	62 (18)	58 (1)	154 (31)	0.67
	24	43 (15)	70 (9)	268 (71)	0.79
	28	26 (4)	46 (10)	192 (48)	0.81
	48	22 (1)	59 (4)	148 (16)	0.78
	52	21 (5)	61 (13)	143 (12)	0.77
GMS	3	31 (5)	53 (10)	211 (15)	0.80
	6	40 (15)	49 (7)	171 (37)	0.75
	24	35 (16)	54 (6)	221 (89)	0.80
	28	40 (7)	59 (1)	186 (14)	0.76
	48	37 (16)	56 (2)	193 (37)	0.77
	52	40 (6)	49 (2)	181 (1)	0.76
PGMS	3	56 (21)	74 (10)	188 (34)	0.71
	6	36 (10)	46 (6)	156 (17)	0.75
	24	26 (10)	65 (21)	345 (166)	0.87
	28	35 (6)	43 (5)	189 (3)	0.79
	48	63 (14)	103 (4)	360 (55)	0.78
	52	74 (42)	105 (13)	401 (19)	0.78

[a] Mold was sequentially cultured in YES and PMS and then transferred in 2-g (wet weight) portions to 50-ml flasks containing 15 ml of GMS, PMS, or PGMS.
[b] Values = $\bar{x}$ (± standard error of the mean); $n \geq 2$ replicate cultures.
[c] Energy charge = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP].

TABLE 3. Aflatoxin production by *A. parasiticus* after transfer to modified replacement media[a]

Modified replacement culture medium	Posttransfer incubation time (h)	Mycelium dry wt (mg)	pH[b]	Amt of aflatoxin in culture (μg)
PMS	1	300	4.7	3.7
	2	310	4.7	3.6
	4	250	4.7	3.0
	6	320	4.9	2.4
	24	290	6.6	2.9
	30	380	7.1	3.0
GMS	1	210	4.6	2.9
	2	280	4.4	2.5
	4	340	4.1	3.7
	6	350	4.1	3.2
	24	320	2.8	8.9
	30	300	2.6	14.8
PGMS	1	270	4.5	3.4
	2	300	4.5	3.3
	4	NT[c]	NT	NT
	6	240	4.4	2.3
	24	390	4.2	16.7
	30	540	4.0	26.2

[a] Mold was sequentially cultured in YES and PMS and then transferred in 5-g (wet weight) portions to 125-ml flasks containing 25 ml of medium modified for NMR studies by omitting KH_2PO_4 and altering the trace metals content (see text).
[b] Initial pH was 4.5.
[c] NT, Not tested.

cultures at various times after transfer from PMS to GMS indicated that low levels of de novo aflatoxin synthesis could be detected as early as 8 to 10 h posttransfer (data not shown). These results are in agreement with those of Abdollahi and Buchanan (1) and indicate that in the replacement culture system employed here the transcriptional and translational processes associated with this de novo gene expression occur after 3 to 6 and 6 to 10 h, respectively, while the initiation of aflatoxin synthesis occurs after 8 to 10 h. Identification of these time periods subsequently allowed specific posttransfer incubation periods to be evaluated with regard to cellular energy status and its effect on the regulation of aflatoxin synthesis.

Energy charge. In previous studies it has been reported that there are apparent differences in energy charge between aflatoxigenic and nonaflatoxigenic strains of *A. parasiticus* and *A. flavus*, respectively (14), and between energy charge and aflatoxin production in zinc-replete and zinc-deficient cultures of *A. parasiticus* (13). Rao et al. (21) have correlated the onset of aflatoxin synthesis in *A. parasiticus* with a reduction in ATP levels and a lowering of energy charge from 0.80 in exponential-phase cells to 0.50 in stationary-phase cells. However, Failla and Niehaus (12) have found no correlation between versicolorin A synthesis and energy charge or total adenine nucleotide content in zinc-replete and zinc-deficient cultures of a blocked mutant of *A. parasiticus*.

In this study energy charge values for mycelia from the three replacement media were similar and remained relatively constant over the course of the posttransfer incubation (Table 2). The average values for the total incubation period were 0.76, 0.77, and 0.78 for PMS, GMS, and PGMS, respectively, which are in agreement with the values reported by Rao et al. (21) for exponential-phase cells. While fluctuations in nucleotide levels were noted among the different media, no pattern could be deduced with regard to the ability of the media to support aflatoxin production. Unlike Rao et al. (21), neither a substantial decline in ATP levels nor changes in energy charge could be correlated with the initiation of during the aflatoxin synthesis. Instead, the data indicate that changes in the relative concentrations of adenosine mono-, di-, and triphosphates are not directly involved in the bioregulation of aflatoxin synthesis.

The differences between the results of this and previous studies (13, 14, 21) likely are due to two reasons. First, the low energy charge value reported by Rao et al. for stationary phase cultures suggests that there is a significant portion of senescing cells, a situation that was avoided in this study by maintaining the mold in a filamentous form. Second, results of preliminary studies in our laboratory indicate that the use of liquid nitrogen-frozen or freeze-dried mycelia in a manner similar to that of Rao et al. (21) resulted in reduced levels of recovery of ADP and ATP accompanied by elevated levels of AMP. This was particularly evident in stationary-phase cells which appeared to have high levels of enzymatic activity leading to the hydrolysis of ATP. This difficulty was avoided by immediately extracting fresh mycelia in conjunction with a high-pressure liquid chromatographic separation and quantitation technique.

^{31}P NMR studies. The influence of cellular energy status on the regulation of aflatoxin synthesis was evaluated further by using in situ ^{31}P NMR analysis. This technique allowed the status of various mobile high-energy phosphorous compounds within mycelia from replacement cultures to be monitored qualitatively in a noninvasive manner. These studies required modification of the replacement media to eliminate KH_2PO_4 and $MnSO_4$ and to reduce the level of other

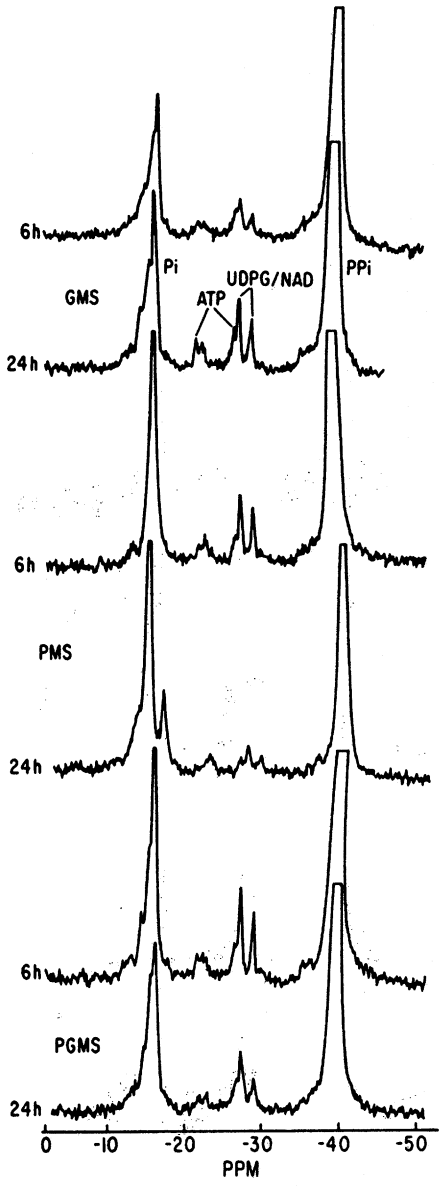

FIG. 2. In situ high-resolution ^{31}P NMR spectra of mycelia after 6 and 24 h of posttransfer incubation in GMS, PMS, and PGMS. Chemical shifts were externally referenced to that of HMPA (data not shown), which was assigned a value of 13.78 ppm. UDPG, UDP-glucose.

mineral salts to prevent paramagnetic broadening and subsequent interference with the detection of intracellular phosphate and phosphate-containing compounds. These modifications did not change the pattern of aflatoxigenesis (Table 3), which was similar to that observed with the unmodified media (Table 1).

Sample evaluations were limited 5,000 transients per accumulation (approximately 13.5 min) after preliminary trials indicated that longer accumulations result in alterations in the pattern of phosphorous compounds due to depletion of available oxygen in the NMR sample tube. Results of additional preliminary studies with uninoculated media indicated that the peptone-containing replacement media (PMS and PGMS) has detectable levels of phosphate (P_i) associated with the peptone source. No attempt was made to remove this low level of P_i because it, in conjunction with media pH determinations, proved useful in identifying intra- and extracellular P_i pools.

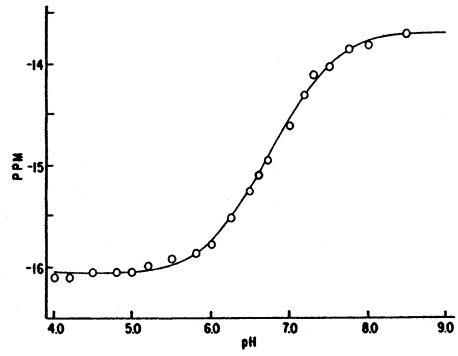

FIG. 3. Standard curve of pH versus ^{31}P chemical shifts of P_i used to estimate intracellular pH.

Representative spectra obtained with mycelia from the three replacement culture media after 6 and 24 h of posttransfer incubation are presented in Fig. 2. The 6-h cultures characteristically had strong signals at chemical shift positions corresponding to P_i(−13 to −16 ppm) and PP_i. Additionally, discernible peaks were observed that corresponded to ATP, UDP-glucose, and NAD. The spectra that were observed with mycelia samples after 0, 1, 2, and 4 h of posttransfer incubation were similar to those for 6 h. Shifts in the magnitude of the detectable peaks compared with that of the HMPA standard were observed after 24 h, with even greater changes being observed after 30 h (data not shown). When the results from aflatoxin-supporting and -nonsupporting replacement cultures were compared, however, no relationship between ^{31}P NMR profiles and aflatoxin production was apparent. Likewise, evaluation of samples obtained from earlier sampling times (0 to 6 h) did not yield any apparent relationship among phosphorous profiles and the induction of aflatoxin synthesis.

^{31}P NMR analysis can also be employed to estimate the pH of various intracellular compartments based on the chemical shifts for P_i (18) (Fig. 3). Multiple P_i pools were apparent in most mycelia samples over the course of the

TABLE 4. Chemical shifts[a] of major P_i peaks detected by ^{31}P NMR analysis of mycelia after incubation in replacement media

Replacement medium[b]	Chemical shifts (ppm) at the following posttransfer incubation times (h):					
	1	2	4	6	24	30
PMS	−16.08	−16.05	−16.05	−16.05	−16.33	−16.40
					−14.24	−14.08
PGMS	−16.03	−16.10	−16.00	−16.08	−16.27	−16.29
				−14.46	−14.75	−14.20
GMS	−15.98	−16.36	−16.06	−16.10	−16.23	−16.31
	−15.63	−16.18	−15.34	−15.42	−15.74	−15.73
		−15.58	−14.40	−14.60	−14.78	

[a] Chemical shifts were externally referenced to that of HMPA, which was assigned a value of 13.78 ppm.

[b] Mold was sequentially cultured in YES and PMS and was then transferred in 5-g portions (wet weight) to 125-ml flasks containing 25 ml of modified replacement medium.

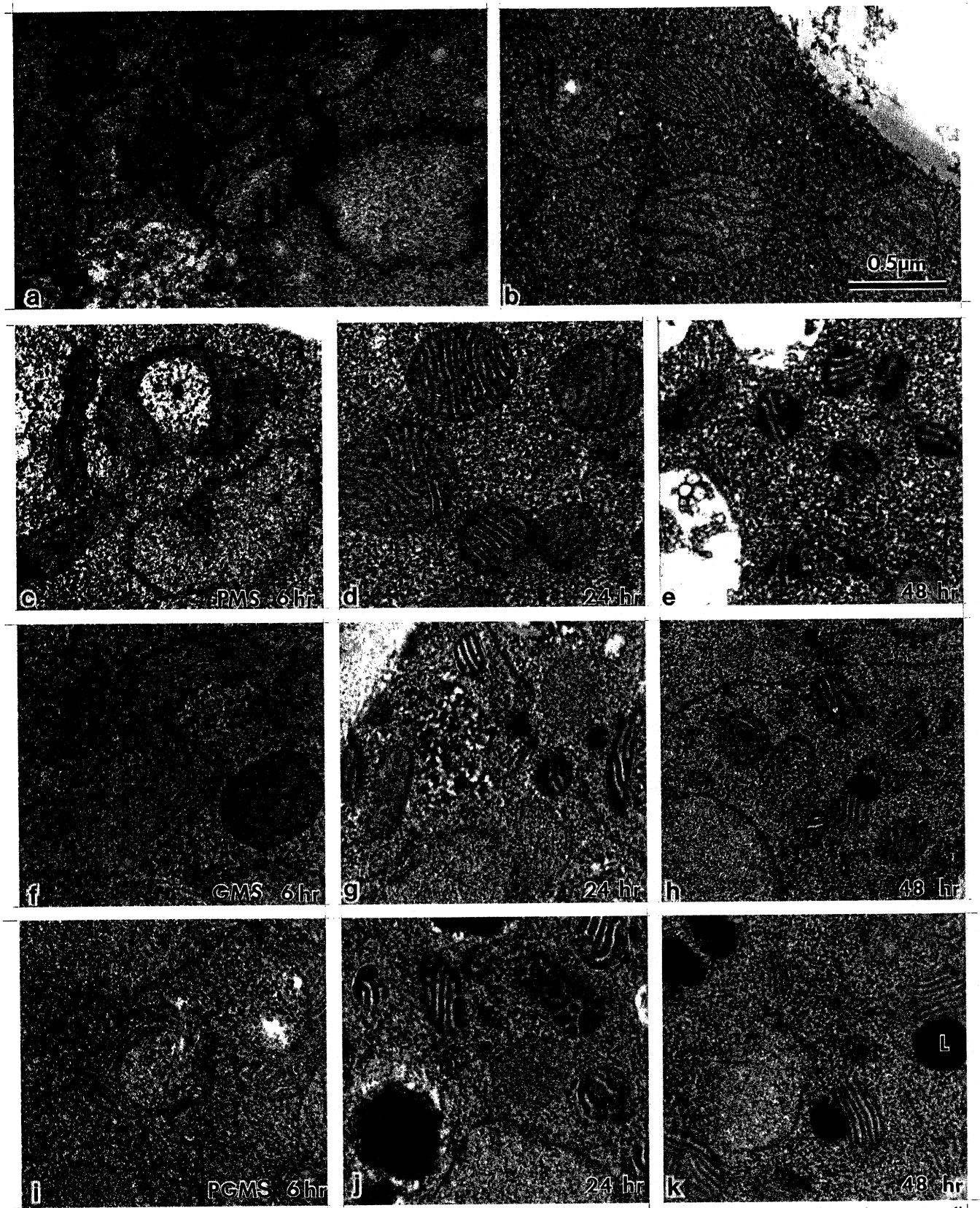

FIG. 4. Changes in mitochondria of *A. parasiticus* after transfer of mycelia to aflatoxin-supporting and -nonsupporting replacement media. Conidia-initiated mycelia that were grown in YES for 72 h (28°C, shaken at 150 rpm) (a). were disrupted and suspended in PMS for 24 h (28°C, shaken at 150 rpm) (b). Portions of the PMS-incubated mycelia were then transferred and incubated (28°C, not shaken) in PMS (nonsupporting) (c to e), GMS (supporting) (f to h), and PGMS (supporting) (i to k) and were sampled at 6, 24, and 48 h. Magnification, × 5,000. L, Lipid.

posttransfer incubation. The chemical shifts of the clearly discernible peaks are summarized in Table 4. Additional P_i peaks were often also apparent, but because of the short accumulation period and limited resolution they could not be definitively assigned a chemical shift value. Adequate resolution of these peaks will require the development of an appropriate gas perfusion system to prevent oxygen depletion so that longer accumulation periods can be achieved. The results of this study suggest that the fungal cells actively maintain vigorous pH gradients; however, no relationship between intracellular pH changes and the induction and subsequent synthesis of aflatoxin is apparent.

The ^{31}P NMR analyses must be considered as preliminary in nature and as being incapable of detecting small changes in intracellular phosphorous compounds. However, the results were consistent with those of the adenylate determinations and suggest further that there is no direct relationship between overall energy status and the bioregulation of aflatoxin synthesis.

Electron microscopy. Changes in mitochondrial ultrastructure were followed in situ throughout the replacement culture protocol (Fig. 4). Mitochondria in cells grown from conidia in YES medium for 72 h with shaking (Fig. 4a) were short ellipsoids. Some regions of mitochondrial membrane, especially in the cristae, were not distinguishable as bilayer structures. When these cells were washed, disrupted, and incubated in PMS for 24 h with shaking (Fig. 4b), mitochondrial sizes were unchanged but the membranes appeared to be better organized. Portions of this homogeneous, PMS-conditioned mycelium were suspended in PMS, GMS, and PGMS; and subsequent changes in mitochondria were followed. Because aflatoxin-supporting cultures produce more toxin when they are not shaken (23) and because the replacement cultures had the physical consistency of mush, no attempt was made to aerate these cultures. The mitochondrial changes that were observed were thus due in part to partial anoxia. However, media-specific changes were also observed.

After 6 h in PMS replacement medium (Fig. 4c), the volume of the mitochondria contracted. The presence of some long profiles suggests that simple elliptical forms do not predominate and that elongation or fusion of mitochondria occurs. In GMS mitochondria appeared as long, relaxed forms (Fig. 4f) in which distinguishable cristae occupied less than half of the apparent mitochondrial volume. Mitochondria in PGMS (Fig. 4i) resembled those in PMS. The mitochondria were contracted; and long forms, some of them circular, were observed. The results indicate that an increase in the length of mitochondria is a response to medium replacement in all three cultures and that the media containing peptone induces moderate mitochondrial contraction by 6 h.

At 24 h mitochondria in PMS-incubated cells (Fig. 4d) were more expanded in volume than at 6 h in the same medium. The mitochondria were spherical and had increased their complement of cristae. In GMS cultures at 24 h (Fig. 4g), the presence of glucose together with anoxic cultural conditions produced apparently degenerate mitochondria that were characterized by extreme contraction and a dense, heavy-staining matrix. Cells in PGMS at 24 h presented a similar mitochondrial morphology (Fig. 4j), but somewhat less severe than that shown in Fig. 4g. Many mitochondria remained large, and the degree of matrix contraction was less than that in GMS. By 48 h, when the lack of oxygen probably became the controlling factor, cells in all three media contained degenerate mitochondria (Fig. 4e, h, and k). In GMS and PGMS cultures, some mitochondria appeared to dissolve in lipid droplets.

We conclude that GMS and PGMS have similar effects on mitochondrial structure that become evident by 24 h. The severe mitochondrial condensation appeared to be due to the presence of glucose and was indicative of degeneration of the organelle. This seemed to be particularly aggravated in replacement cultures in which the partially anoxic conditions appeared to amplify the effect. Inactivation of the mitochondria due to the presence of glucose agrees with results of previous investigations (4, 6) in which the bioregulation of aflatoxin synthesis was correlated with an apparent catabolite repression of tricarboxylic acid cycle activity.

The results of the adenylate energy charge and ^{31}P NMR analyses suggest that the induction of aflatoxin synthesis by carbohydrates (i.e., glucose) is not controlled by the overall energy status of the fungal cell. Examination of mitochondrial morphology in aflatoxin-supporting and -nonsupporting replacement cultures, however, indicates that carbohydrate-associated inactivation of the principal organelle is responsible for energy generation. This implies that a secondary site for energy generation is responsible for maintenance of the energy status of the cell. This suggests further that a shift to an extramitochondrial source of energy may be involved in the bioregulation of aflatoxin synthesis and that the production of this group of secondary metabolites may be influenced by the energy status of specific subcellular compartments.

LITERATURE CITED

1. **Abdollahi, A., and R. L. Buchanan.** 1981. Regulation of aflatoxin biosynthesis: characterization of glucose as an apparent inducer of aflatoxin production. J. Food Sci. **46**:143–146.
2. **Abdollahi, A., and R. L. Buchanan.** 1981. Regulation of aflatoxin biosynthesis: induction of aflatoxin production by various carbohydrates. J. Food Sci. **46**:633–635.
3. **Applebaum, R. S., and R. L. Buchanan.** 1979. Intracellular concentrations of cyclic AMP and cyclic GMP in an aflatoxigenic strain of *Aspergillus parasiticus*. J. Food Sci. **44**:116–117, 122.
4. **Buchanan, R. L., D. Federowicz, and H. G. Stahl.** 1985. Activities of tricarboxylic acid cycle enzymes in an aflatoxigenic strain of *Aspergillus parasiticus* after a peptone to glucose carbon source shift. Trans. Br. Mycol. Soc. **84**:267–275.
5. **Buchanan, R. L., and W. M. Houston.** 1982. Production of blue-fluorescent pyrazines by *Aspergillus parasiticus*. J. Food Sci. **47**:779–782.
6. **Buchanan, R. L., and D. F. Lewis.** 1984. Regulation of aflatoxin biosynthesis: effect of glucose on the activities of various glycolytic enzymes. Appl. Environ. Microbiol. **48**:306–310.
7. **Buchanan, R. L., L. A. Ocker, and H. G. Stahl.** 1985. Effect of 2-deoxyglucose, alpha-methyl glucoside, and glucosamine on aflatoxin production by *Aspergillus parasiticus*. Arch. Microbiol. **142**:200–203.
8. **Buchanan, R. L., and H. G. Stahl.** 1984. Ability of various carbon sources to induce and support aflatoxin synthesis by *Aspergillus parasiticus*. J. Food Safety **6**:271–279.
9. **Davis, N. D., and U. L. Diener.** 1968. Growth and aflatoxin production by *Aspergillus parasiticus* from various carbon sources. Appl. Microbiol. **16**:158–159.
10. **Davis, N. D., U.L. Diener, and V. P. Agnihotri.** 1967. Production of aflatoxins B_1 and G_1 in a chemically defined medium. Mycopathol. Mycol. Appl. **31**:251–256.
11. **Davis, N. D., U. L. Diener, and D. W. Eldridge.** 1966. Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. Appl. Microbiol. **14**:378–380.
12. **Failla, L. J., and W. G. Niehaus, Jr.** 1986. Regulation of Zn^{2+} uptake and versicolorin A synthesis in a mutant strain of

Aspergillus parasiticus. Exp. Mycol. **10**:35–41.
13. **Gupta, S. K., K. K. Maggon, and T. A. Venkitasubramanian.** 1976. Effect of zinc on adenine nucleotide pools in relation to aflatoxin biosynthesis in *Aspergillus parasiticus*. Appl. Environ. Microbiol. **32**:753–756.
14. **Gupta, S. K., K. K. Maggon, and T. A. Venkitasubramanian.** 1977. Regulation of aflatoxin biosynthesis. III. Comparative study of adenine nucleotide pool in aflatoxigenic and non-aflatoxigenic strains. Microbios **19**:37–44.
15. **Hsieh, D. P. H., and R. I. Mateles.** 1971. Preparation of labelled aflatoxins with high specific activities. Appl. Microbiol. **22**:79–83.
16. **Khym, J. X.** 1975. An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. Clin. Chem. **21**:1245–1252.
17. **Mateles, R. I., and J. C. Adye.** 1965. Production of aflatoxins in submerged culture. Appl. Microbiol. **13**:208–211.
18. **Moon, R. B., and J. H. Richards.** 1973. Determination of intracellular pH by ^{31}P magnetic resonance. J. Biol. Chem. **248**:7276–7278.
19. **Pfeffer, P. E., S. Tu, W. V. Gerasimowicz, and J. R. Cavanaugh.** 1985. In vivo ^{31}P NMR studies of corn root tissue and its uptake of toxic metals. Plant Physiol. **80**:77–84.
20. **Prasad, R. B.** 1983. Influence of carbon sources on growth and aflatoxin production by *Aspergillus parasiticus* (NRRL 3240), p. 207–215. *In* K. S. Bilgrami, T. Prasad, and K. K. Sinnha (ed.), Mycotoxins in food and feed. Allied Press, Bhagalpur, India.
21. **Rao, V. M., S. Saraswathy, K. K. Maggon, and T. A. Venkitasubramanian.** 1980. Bioenergetics of aflatoxin biosynthesis in *Aspergillus parasiticus*. J. Food Sci. **45**:1031–1035.
22. **Roberts, J. K. M., D. Wemmer, P. M. Ray, and O. Jardetzky.** 1982. Regulation of cytoplasmic and vacuolar pH in maize root tips. Plant Physiol. **69**:1344–1347.
23. **Shih, C. N., and E. H. Marth.** 1974. Aflatoxin formation, lipid synthesis, and glucose metabolism by *Aspergillus parasiticus* during incubation with and without agitation. Biochem. Biophys. Acta **338**:286–296.
24. **Shih, C. N., and E. H. Marth.** 1974. Some cultural conditions that control biosynthesis of lipid and aflatoxin by *Aspergillus parasiticus*. Appl. Microbiol. **27**:452–456.